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SHOWDOMYCIN AND ITS REACTIVE MOIETY, MALEIMIDE

A COMPARISON IN SELECTIVE TOXICITY AND MECHANISM OF ACTION *IN VITRO**

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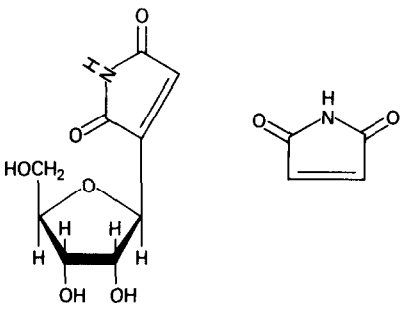
Abstract—Showdomycin, a C-nucleoside antibiotic, was twice as toxic to L1210 murine leukemia cells as to murine bone marrow progenitor cells of the granulocyte-macrophage series. Its aglycone, maleimide, was equally toxic to both cell lines. Cysteine, adenosine and the potent nucleoside transport inhibitor 6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (HNBMPR) reversed the early stages of toxicity of showdomycin to L1210 cells, but did not reduce the toxicity of maleimide. At cytotoxic concentrations, showdomycin progressively inactivated the nucleoside uptake system to completion. This inhibition of nucleoside uptake was reversed by cysteine under conditions where it reversed cytotoxicity. The binding of 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) by L1210 cells was also inhibited by showdomycin, indicating that the antibiotic inactivated the nucleoside transport site. The data suggest that the C-nucleoside structure confers some selectivity to the cytotoxic action of maleimide, directing it toward the nucleoside transport system of the tumor cell.

Showdomycin (Fig. 1) is a C-nucleoside antibiotic whose activity is due to the alkylating property of its maleimide moiety [1, 2]. It is active against both gram positive and gram negative bacteria [2], HeLa cells *in vitro* and the Ehrlich ascites carcinoma *in vivo* [3]. The transport of showdomycin in *Escherichia coli* has been shown to be similar to that of a wide variety of nucleosides [4]. Its mechanism of action may, therefore, be explained by both its uptake as a nucleoside analog and its reactivity as a maleimide sulfhydryl reagent. We here report on two aspects of showdomycin cytotoxicity to mammalian cells: (1) a comparison between the C-nucleoside and its maleimide moiety with regard to selective toxicity toward L1210 tumor cells and a normal renewal system, the macrophage–granulocyte progenitor cells of the bone marrow, and (2) the specificity of its mechanism of action.

MATERIALS AND METHODS

Materials. Bovine serum albumin was obtained as serum fraction V from Miles Laboratories, Elkhart, IN. Fetal calf serum was purchased from Flow Laboratories, Rockville, MD, and RPMI 1630 medium, Dulbecco's phosphate-buffered saline, penicillin and streptomycin were supplied by the NIH Media Unit. Modified McCoy's 5A medium was obtained from Grand Island Biological Co., Grand Island, NY. Showdomycin was supplied by Dr. John Douros, of the Natural Products Branch of the Developmental Therapeutics Program, DCT, NCI, and was prepared as a stock solution of 20–40 mM in cold distilled water. Maleimide, a product of the Aldrich Chemical Co., Inc, Milwaukee, WI, was dissolved in chilled distilled water to a concentration of 200 mM before use. Pregnant mouse uterine extract, a source of colony stimulating factor (CSF) in the bone marrow assays [5], was a gift of Dr. T. R. Bradley of the Cancer Institute, Melbourne, Australia, and of Dr. Richard Knazek of the Laboratory of Pathophysiology, NCI, Bethesda, MD. [5,6-^{3}H]Uridine (47 Ci/mmole) and [2,8-^{3}H]adenosine (36.7 Ci/mmole) were purchased from New England Nuclear, Boston, MA. They were diluted with the respective unlabeled nucleosides and used at concentrations indicated in the text. [G-^{3}H]Nitrobenzylthioinosine (NBMPR)§ was obtained from Moravek Biochemicals, City of



SHOWDOMYCIN MALEIMIDE

Fig. 1. Structures of showdomycin and maleimide.

* A preliminary report of this work has been presented [Y. Uehara, J. M. Fisher and M. Rabinovitz, *Proc. Am. Ass. Cancer Res.* **20**, 239 (1979)].

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§ Abbreviations used are: HNBMPR, 6-[2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; and NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine.

Industry, CA, as a solution of 20 Ci/mmole in methanol. The silicone oil, Versilube F-50, was obtained from the Harwick Chemical Corp., Cambridge, MA.

Tumor cell cytotoxicity. L1210 cells were grown in RPMI 1630 medium supplemented with 16% heat-inactivated fetal calf serum. These were harvested in the logarithmic phase of growth ($5–10 \times 10^5$ cells/ml), washed twice by suspension and centrifugation in Dulbecco's phosphate-buffered saline (PBS) containing 0.1 mM bovine serum albumin and 0.25% glucose (PAG), and suspended at 10^5 cells/ml in the same buffer system. Showdomycin or maleimide was then added and the incubation continued for 30 min. Cells were harvested by centrifugation, resuspended in growth medium containing 40 μg/ml gentamicin (Schering), and washed two additional times by suspension in this medium and centrifugation. Cytotoxicity was assessed either by clonal growth of surviving cells for 2 weeks in soft nutrient-agar according to the procedure of Chu and Fischer [6] with minor modifications [7], or by static growth for 45 hr in RPMI 1630 supplemented with 16% heat-inactivated fetal calf serum and containing 40 μg/ml gentamicin.

Bone marrow cytotoxicity. Male CDF_1 mice, weighing 20–25 g, were killed by cervical dislocation, and the femurs were removed and gently flushed with PAG. The contents were washed twice with this medium and suspended in it at 10^5 nucleated cells/ml. The experimental protocol for exposure to showdomycin or maleimide was identical to that described above for L1210 cells. Toxicity to murine hematopoietic precursor cells was assessed after clonal growth for 1 week in modified McCoy's 5A medium supplemented with 15% fetal calf serum, 20 units/ml penicillin and 20 μg/ml streptomycin, in a humidified atmosphere of 10% carbon dioxide [8].

Pregnant mouse uterine extract was used at a concentration that resulted in maximal colony formation of 90–100 macrophage and granulocyte colonies per 100,000 nucleated bone marrow cells; no colony formation occurred in its absence. Cell aggregates were scored as colonies if they contained 50 or more cells.

Uptake of nucleosides by L1210 *cells.* Logarithmic phase L1210 cells were harvested by centrifugation at 300 *g* for 5 min and washed twice with transport medium composed of Dulbecco's PBS containing 0.1 mM bovine serum albumin and 0.1% glucose. They were than suspended at a concentration of 10^5 cells/ml. Uptake was either initiated by simultaneous addition of labeled nucleosides and showdomycin, maleimide or water as a control, or cells were first treated with showdomycin and maleimide for the times indicated, washed twice, suspended at a concentration of 10^6 cells/ml and incubated with labeled nucleosides. Aliquots of the incubation mixture were layered on Versilube F-50 silicone oil in a microcentrifuge tube and uptake was terminated by centrifugation of the cells through the oil at 12,000 *g* for 1 min in an Eppendorf microcentrifuge. Tips containing the cell pellet were cut off, the pellets were solubilized in 0.2 M NaOH and neutralized with acetic acid, liquid scintillation fluor was added, and samples were counted on a Beckman liquid scintillation counter. Uptake estimates were performed in duplicate.

NBMPR binding assay. Logarithmic phase L1210 cells were harvested and washed twice with PAG and suspended in transport medium at a concentration of 10^6 cells/ml. Binding assays were initiated by adding [^{3}H]-NBMPR, and assay intervals were terminated by centrifuging 200μl samples through silicone oil at 12,000 *g* for 1 min. The cell pellets were assayed for associated radioactivity as described above.

RESULTS

Cytotoxicity of showdomycin and maleimide to L1210 *and bone marrow progenitor cells.* Showdomycin was twice as toxic as maleimide to L1210 cells,

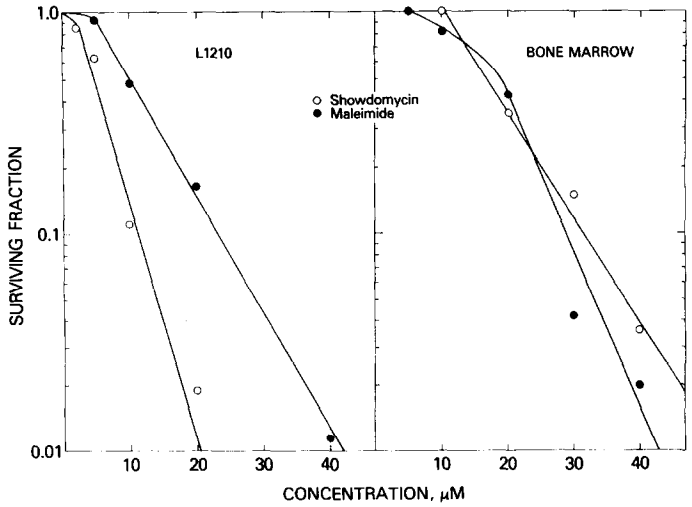

Fig. 2. Cytotoxicity of showdomycin and maleimide toward L1210 and bone marrow progenitor cells. Cell suspensions (10^5 cells/ml), prepared as described in Materials and Methods, were incubated for 30 min in PAG at 37° with the indicated concentrations of showdomycin (○) or maleimide (●). They were then harvested and washed, and the toxicity of showdomycin and maleimide was estimated by clonal growth, as described in the text.

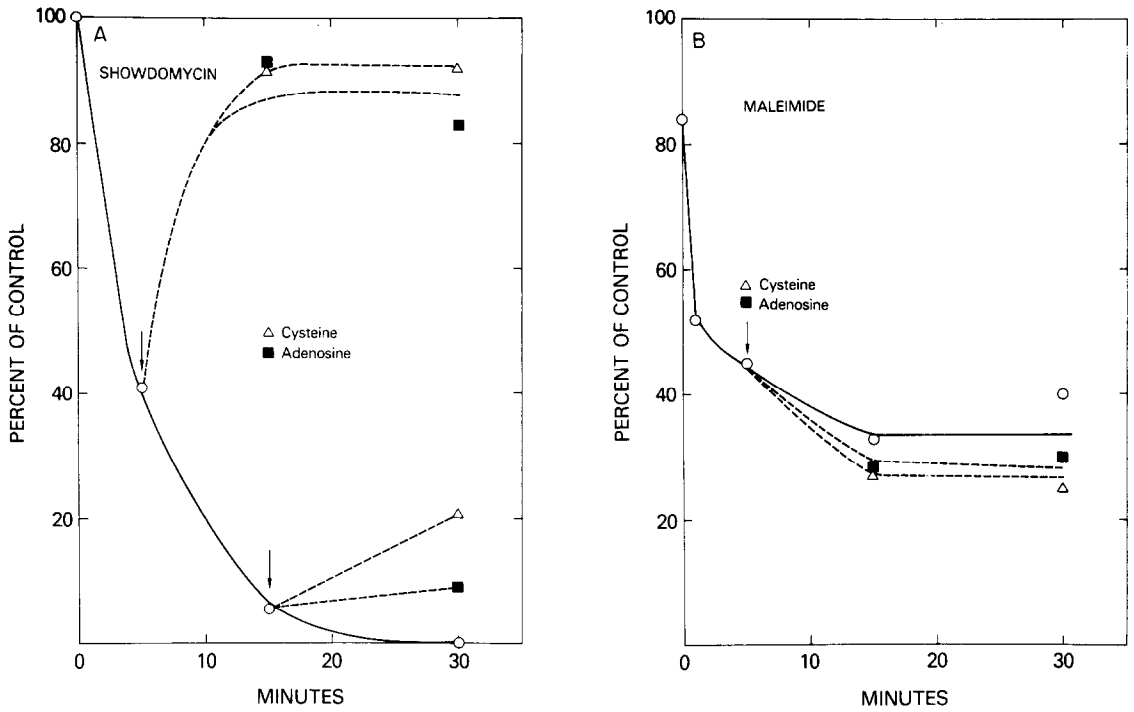

Fig. 3. Onset and reversibility of showdomycin (A) and maleimide (B) cytotoxicity to L1210 cells. Cell suspensions (10^5 cells/ml) were incubated with showdomycin (20 μM) or maleimide (20 μM). Cysteine (50 μM) (△) or adenosine (2 mM) (■) was added as indicated. Cells were washed twice with RPMI 1630 growth medium and toxicity was estimated by growth in static culture for 45 hr.

but the two compounds were equally toxic to bone marrow cells (Fig. 2). This indicates that the nucleoside carrier moiety contributed selective toxicity toward the tumor cell type.

Reversal of showdomycin and maleimide cytotoxicity by cysteine, adenosine and HNBMPR. When L1210 cells were incubated with showdomycin at 20 μM, cytotoxicity progressed rapidly as shown in Fig. 3A. Toxicity was reversed when cysteine or adenosine was added, only during its early stages (Fig. 3A, Table 1). HNBMPR, a specific nucleoside transport inhibitor [9, 10], also reversed showdomycin toxicity, but adenine did not (Table 2). If cells were first incubated with these compounds, except for adenine, showdomycin cytotoxicity was abolished completely (data not shown).

Maleimide cytotoxicity showed a different response to cysteine and adenosine. If cysteine were present before maleimide addition, no cell kill was observed and, as expected, adenosine offered no protection from maleimide (data not shown). The reversal of cytotoxicity by these compounds was not observed even during the early stages of inhibition (Fig. 3B, Table 1). Similar results were obtained with bone marrow cells (data not shown). In these assays, cysteine (50 μM), adenosine (2 mM) or HNBMPR (10 μM) did not affect the growth of either L1210 or bone marrow cells.

Effect of showdomycin and maleimide on the nucleoside uptake system. When showdomycin and labeled uridine were added simultaneously to L1210 cells, uridine uptake was normal for the first few

Table 1. Reversal of showdomycin cytotoxicity to L1210 cells as determined by clonal growth

Addition at zero time	Additions at 5 min	Per cent survival*	
		5 min	15 min
Showdomycin (20 μM)		33	4
	Cysteine (50 μM)		74
	Adenosine (2 mM)		60
Maleimide (20 μM)		32	35
	Cysteine (50 μM)		35
	Adenosine (2 mM)		32

* Cells were incubated as indicated in the legend of Fig. 3. Clonal growth was estimated as described in Materials and Methods.

Table 2. Reversal of showdomycin cytotoxicity to L1210 cells

Addition at zero time	Additions at 5 min	Cell proliferation rate* (% of control) 5 min	15 min
Showdomycin (20 μM)		20	0
	Cysteine (50 μM)		70
	Adenosine (1 mM)		53
	HNBMPR (10 μM)		37
	Adenine (1 mM)		3

* Population doublings in 45 hr as a percentage of rates in cultures without additives (3.5 doublings in 45 hr).

minutes, but inhibition developed progressively and later uptake was blocked completely (Fig. 4). Maleimide, on the other hand, inhibited uridine uptake partially and at a constant rate throughout the incubation period (Fig. 4).

Prior exposure of L1210 cells to showdomycin, followed by washing and incubation in drug-free medium, resulted in almost complete inactivation of the uridine (Fig. 5) and adenosine (Fig. 6) uptake systems. An equitoxic concentration of maleimide did not inactivate uridine uptake (Fig. 5). The inactivation of adenosine uptake by showdomycin was partially reversed by the addition of cysteine (Fig. 7).

Inactivation of the nucleoside transport site by showdomycin. To determine whether showdomycin inhibition of nucleoside uptake was due to inactivation of the nucleoside transport site [11] or to inhibition of subsequent metabolism [12], the effect of the antibiotic on NBMPR binding was studied. This nucleoside has been shown by Lauzon and Paterson [13] to bind specifically to nucleoside transport sites of the plasma membrane. As shown in Fig. 8, cells incubated with showdomycin were inhibited in binding of NBMPR, indicating that the antibiotic inactivated the nucleoside transport sites.

DISCUSSION

Komatsu and Tanaka [14] found that both showdomycin and *N*-ethylmaleimide inhibited deoxyribonucleotide syntheses equally in *E. coli*, and Roy-Burman and Visser [15] reported that in this organism the inhibition of glucose and nucleoside uptake by showdomycin and *N*-ethylmaleimide occurred at

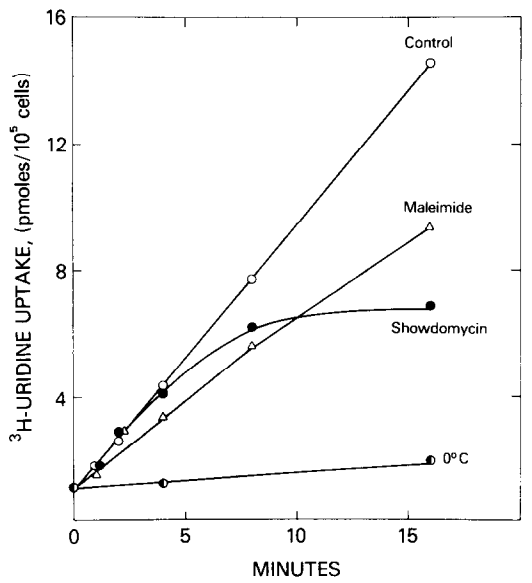

Fig. 4. Effects of showdomycin and maleimide on uridine uptake by L1210 cells. Cell suspensions (10^5 cells/ml) were incubated at 37° in transport medium containing [5,6-^{3}H]uridine (1.0 μM) and either showdomycin (20 μM) (●), maleimide (20 μM) (△), or an equivalent volume (10 μl) of water (○) as control. Uridine uptake was also followed at 0° (◐) and was estimated as described in Materials and Methods.

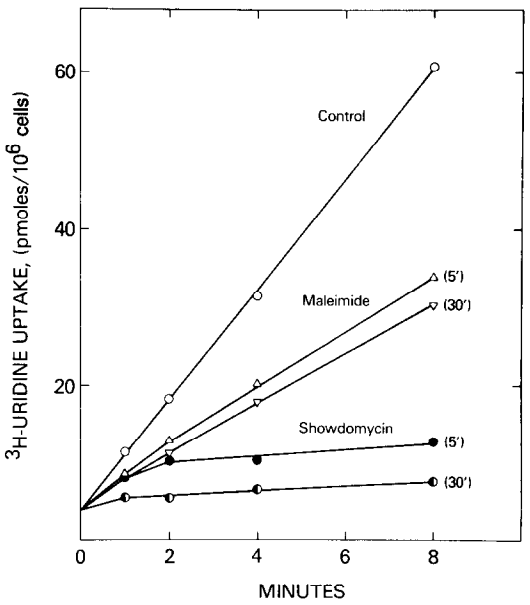

Fig. 5. Inactivation on uridine uptake by showdomycin. L1210 cell suspensions (10^5 cells/ml) were incubated in PAG with showdomycin (20 μM) for 5 min (●) or 30 min (◐); maleimide, (20 μM) for 5 min (△) or 30 min (▽) or with an equivalent volume (10 μl) of water (○) as control. Cells were harvested by centrifugation, washed with transport medium, and resuspended at 1×10^6 cells/ml. [5,6-^{3}H]Uridine was added to a final concentration of 10 μM, and uptake was estimated as described in Materials and Methods.

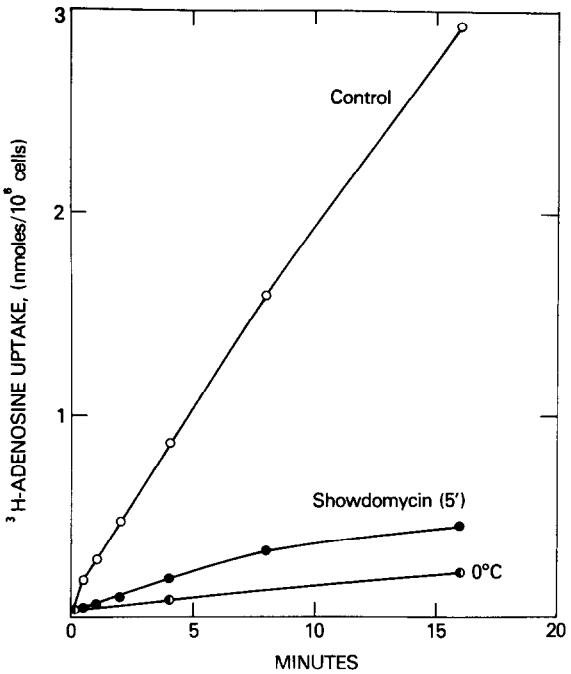

Fig. 6. Inactivation of adenosine uptake by showdomycin. L1210 cell suspensions (10^5 cells/ml) were incubated in PAG with showdomycin (20 μM) for 5 min (●) or an equivalent volume (10 μl) of water (○) as control, washed with transport medium, and resuspended at 10^6 cells/ml. [2,8-^{3}H]Adenosine was added to a final concentration of 10 μM, and uptake was estimated as described in Materials and Methods. Adenosine uptake was also followed at 0° (◐).

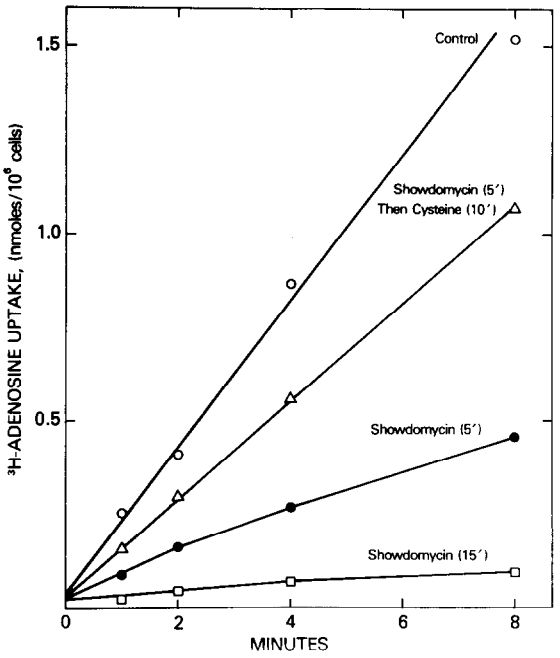

Fig. 7. Recovery of adenosine uptake in cysteine-treated L1210 cells. A cell suspension (10^5 cells/ml) was incubated in PAG with showdomycin (20 μM) for 5 min. Cysteine was added to a final concentration of 50 μM, and the incubation continued for an additional 10 min. Cells were washed with transport medium, and adenosine uptake was compared with that of cells treated with showdomycin for 5 min or 15 min but without subsequent incubation with cysteine. Key: untreated controls (○), showdomycin treatment for 5 min (●) or 15 min (□), and showdomycin for 5 min then cysteine for 10 min (△).

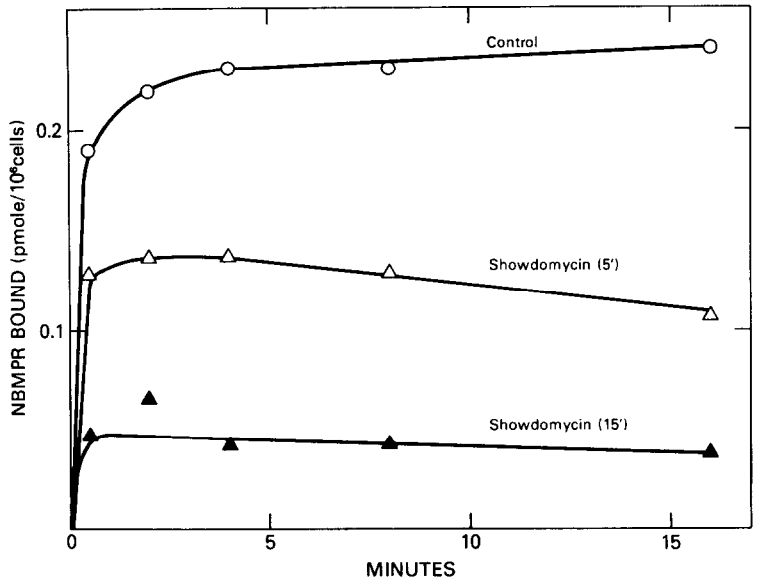

Fig. 8. Inactivation of NBMPR binding by showdomycin. L1210 cell suspensions (10^5 cells/ml) were incubated with showdomycin (20 μM) for 5 or 15 min in PAG at 37°, washed with transport medium, and resuspended at 10^6 cells/ml. [^{3}H]-Nitrobenzylthioinosine was added to a final concentration of 10 nM, and binding was estimated as described in Materials and Methods. Key: untreated controls (○), and showdomycin treatment for 5 min (△) or 15 min (▲).

similar concentrations. Since both compounds exerted their primary inhibitory effect by alkylation of susceptible sulfhydryl groups, it was suggested that showdomycin acted in the same non-specific manner as maleimide and other sulfhydryl reagents. We report a marked difference between the two compounds in mammalian cells, both in toxicity and mechanism of action. Showdomycin has selective toxicity toward L1210 tumor cells when compared to normal bone marrow progenitor cells and shows specificity in inactivating the nucleoside uptake system.

There may exist a difference in the rate or character of alkylation of showdomycin vs maleimide, because cysteine and adenosine reversed the early stage of showdomycin cytotoxicity but not that of maleimide. The protective and reversal effects of adenosine and HNBMPR may be due to competition with showdomycin for initial binding to the transport carrier, and the reaction by cysteine with the maleimide moiety will make it unavailable for interaction with cellular sulfhydryl sites. These results suggest that showdomycin had not yet reacted with cellular sites during the early stages of incubation, while maleimide reacted immediately. The onset of maleimide cytotoxicity was also much more rapid than that of showdomycin (Fig. 3). The slower reaction rate of showdomycin may be due in part to the location of the ribose moiety at the 2 position; however, citraconimide, the 2-methyl analog of maleimide, is much slower to react with cysteine and glutathione than either showdomycin or *N*-ethylmaleimide [16, 17]. The observation may also be due to the low affinity of a C-nucleoside for initial binding to nucleoside transport sites (Fig. 4).

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